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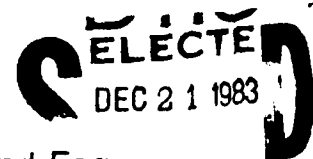
Mg⁺⁺ and K⁺ Distribution in Frog Muscle and Egg: A Disproof of the Donnan Theory of Membrane Equilibrium Applied to the Living Cells

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ABSTRACT   1. We studied the equilibrium distribution of Mg⁺⁺ in the form of chloride and sulfate at two temperatures (5° and 25°C) in frog voluntary muscles. External Mg⁺⁺ concentration was varied between 1.2 and 73.2 mM with the specific purpose of testing the diametrically opposed predictions of the membrane theory and the association-induction hypothesis.

2. There was a linear gain of Mg⁺⁺ over the entire range of external Mg⁺⁺ concentrations studied at both 5°C and 25°C. In a plot of intracellular Mg⁺⁺ concentration in μmoles per gram of fresh muscle cells against extracellular Mg⁺⁺ concentration, the slopes observed were 0.220 at 5°C and 0.0206 at 25°C.

3. The increase of external Mg⁺⁺ from 1.2-73.2 mM at constant external K⁺ concentration (2.5 mM) had no discernible effect on intracellular K⁺ concentration, which remained constant at its normal levels in the vicinity of 90 μmoles/g/ fresh muscle cells.

4. We observed a similar rectilinear distribution of Mg⁺⁺ in frog ovarian eggs. As in muscle tissues, no major alteration of intracellular K⁺ concentration results from increases of external Mg⁺⁺ concentration from 1.2-73.2 mM.

5. With the rectilinear gain of Mg⁺⁺, there was an entirely parallel gain of chloride in frog muscle cells. Indeed the slope of the Cl⁻ curve and that of the Mg⁺⁺ curve have essentially the same value. Thus Mg⁺⁺ has entered the cell accompanied by chloride (and sulfate), rather than by exchange with other intracellular cations.

6. An increase of external K⁺ from 2.5 mM to 100 mM at a constant external Mg⁺⁺ concentration depolarizes the muscle cell resting potential as shown by Ling and Gerard, ('50) and causes an increase of intracellular K⁺, doubling its normal concentration to 200 μmoles/g fresh muscle cells. Notwithstanding, the intracellular concentration of Mg⁺⁺ remained totally unchanged from its normal value.

7. These findings profoundly disagree with the predictions of the Donnan theory of membrane equilibrium, according to which profound alteration of intracellular K⁺ concentration should follow exposure to high external Mg⁺⁺ concentration, and vice versa. Furthermore, the postulation of a Mg⁺⁺ pump is not feasible not only because of its energy requirements, but because it would also be inadequate to explain the lack of effect of varying external Mg⁺⁺ concentration on the resting potential, the intracellular K⁺ concentrations, as well as the pattern of Cl⁻ uptake totally different from that predicted by the membrane theory.

8. On the other hand, Mg⁺⁺, K⁺, and Cl⁻ distributions correlated completely with the predictions of the association-induction hypothesis, according to which Mg⁺⁺ and K⁺, in a normal resting cell, are predominantly adsorbed on sites they do not share, hence there is no mutual interference. Saturating all the intracellular sites at an external concentration of 1.2 mM, the concentration of Mg⁺⁺ in the muscle cells increased further only in the form of free Mg⁺⁺ (accompanied by its anion) in the cell water.

9. The q-value for Mg⁺⁺ in muscle cells at two different temperatures permits calculation of the thermodynamic parameters of the distribution of Mg⁺⁺ salt in frog muscle cell water: a moderately favorable ΔH equal to −0.516 Kcal/mole, and an unfavorable entropy of 4.37 cal/degree/mole, showing an entropic cause for the exclusion of Mg⁺⁺ from cell water.

The basic assumption of the membrane theory, since its founding by Pfeffer in 1877 (Pfeffer, '21), has been that living cells behave as sacs full of an aqueous solution, enclosed and held together by a semipermeable membrane. The low level of cell Na$^+$ was long believed to be due to an impermeability of the cell membrane to this ion; on the other hand, the high level of cell K$^+$, an ion known to penetrate into and out of the cell, was thought to be due to a Donnan membrane equilibrium (Donnan, '24)—a view presented in a coherent and masterful manner by Boyle and Conway in 1941 (Boyle and Conway, '41).

However, even as this landmark publication of Boyle and Conway was being put into print, data were rapidly gathering pointing to the fact that Na$^+$ in fact also enters and leaves cells with apparent ease (Cohn and Cohn, '39; Heppel, '40; Steinbach, '40). To explain this new finding an interpretation by no means new even then (Lillie, '23) was brought to the fore: the low level of cell Na$^+$ is maintained by the ceaseless efforts of postulated Na pumps in the cell membrane (Dean, '41; Krogh, '46). However, as long as one retains the basic assumption of the membrane theory mentioned above, and as long as one continues to accept Donnan's theory of membrane equilibrium, the postulation of the Na pump (and other pumps) would not eliminate the forces described in Donnan's theory. Thus, for example, the excess negative charges carried by impermeant anions in the cell must still be balanced by the intracellular cations largely in the form of K$^+$. In fact, one of Boyle and Conway's key experiments on K$^+$ distribution in frog muscle cells has recently been repeated (Palmer and Gulati, '76) and the results claimed once more to uphold the Donnan theory of membrane equilibrium as the correct explanation for K$^+$ distribution in these cells.

To overcome various difficulties of the membrane theory (Ling, '62; Ling et al., '73; Ling, '78b) including those brought about by the above-mentioned discovery of Na$^+$ permeability to the cell membrane, another alternative would have been to abandon the basic assumption of the membrane theory, that the cell interior is essentially a dilute aqueous solution. Such an approach has been taken, and is most extensively developed in the form of the association-induction hypothesis (Ling, '62). This hypothesis does not view the physical state of cell water and ions as being that of a dilute aqueous solution. Its simple basic con-

cepts provide a ready explanation for the data of Boyle and Conway as well as those of Palmer and Gulati (Ling, '77a).

In this communication we present results of a further test of the Donnan Equilibrium, and hence of the membrane pump theory, vis-a-vis the association-induction hypothesis.

THEORIES

The membrane theory

It is well known that both K$^+$ and Cl$^-$ are permeant to living cells (Boyle and Conway, '41). Recently we have shown that Mg^{++} is also permeant, further confirming earlier unanimous conclusions (Conway and Cruess-Callaghan, '37; Gilbert, '60; Shield and Straub, '68). According to Donnan's membrane theory ('24) the equilibrium distribution of K$^+$, Mg^{++}, and Cl$^-$ follows the following relation

$$\frac{[K^+]_{in}}{[K^+]_{ex}} = \frac{[Cl^-]_{ex}}{[Cl^-]_{in}} = \left\{ \frac{[Mg^{++}]_{in}}{[Mg^{++}]_{ex}} \right\}^{1/2} = r, \qquad (1)$$

where $[\ ]_{in}$ and $[\ ]_{ex}$ refer to intra- and extracellular concentrations, and r is the Donnan ratio. Furthermore the electrical potential difference between these two phases or membrane potential (Ψ) is described by

$$\Psi = \frac{RT}{F} \ln r, \qquad (2)$$

where R and F are the gas and Faraday constant and T, the absolute temperature.

In such a Donnan membrane equilibrium system, *the distribution of all permeant ions are interdependent both on each other and on the membrane potential* Ψ. Given the concentration of impermeant ion R and the amounts of the free ions present, a unique pattern of ion distribution and electrical potential emerges from Equations 1 and 2. When a major change occurs in the external concentration of one of the free ions present, the distribution of all permeant ions as well as Ψ must readjust until another unique pattern is established. This general principle is illustrated in the following, more specific example. Based on the relationships shown in Equation 1 and those shown in following equations,

$$[K^+]_{ex} + 2[Mg^{++}]_{ex} = [Cl^-]_{ex}, \qquad (3)$$

and

$$[K^+]_{in} + 2[Mg^{++}]_{in} = [Cl^-]_{in} + R^-, \qquad (4)$$

one can readily predict the consequences when the external free Mg^{++} concentration is increased from nil to 73.2 mM and is maintained at this high concentration: Thus assuming a value of 120 mEg/liter for the concentration of the impermeant anion, R$^-$, the Donnan

ratio r should plunge from a value of 48 to 1.04; [Cl$^-$]$_{in}$ and free [Mg^{++}]$_{in}$ would rise to 139 mM and 80 mM, respectively; and [K$^+$]$_{in}$ would drop from 120 mM to 2.6 mM.

Conversely, when the external K$^+$ concentration is raised from, say, its normal value of 2.5 mM to 100 mM, the resting potential is known to fall to near zero (Ling, '62; Ling and Gerard, '50); Equations 1, 2, 3, and 4 then predict a considerable decrease in the intracellular free Mg^{++} concentration. It may be mentioned that in 1942 Fenn and Haege reported such a reciprocal change of frog muscle K$^+$ and Mg^{++} (Fenn and Haege, '42). Gilbert ('60) on the other hand, postulated a Mg^{++} pump, with the clear implication that Mg^{++} distribution does not follow Donnan's membrane equilibrium theory.

It is worth mentioning that theoretically the possession of an intact membrane is not absolutely essential for a Donnan equilibrium postulation to be established. The restraint from diffusion of the "impermeant" charged species could be achieved by being part of an immobile matrix, a case discussed by Proctor and Wilson in 1916 (Proctor and Wilson, '16) and more recently by Edwards and coworkers (Collins and Edwards, '71; Pemrick and Edwards, '74).

The association-induction hypothesis

According to the association-induction hypothesis, the living cell does not represent a Donnan membrane system (Ling, '62). The electrical potential of a resting cell, or its resting potential, is determined by the existence of fixed anionic sites on a microscopically thin layer of the cell surface (Ling, '59, '60, '67a, '75; Ling and Walton, '79). The nature and density of these fixed anionic sites (e.g., β- and γ-carboxyl groups), the ionic composition of the surrounding medium, and the temperature determine the magnitude and polarity of Ψ; Ψ does not determine the macroscopic total intracellular ionic concentration nor does the total intracellular ionic concentration determine Ψ.

The general equation for solute distribution according to this hypothesis is (Ling, '65b):

$$[p_i]_{in} = \alpha q_i \, [p_i]_{ex} + \sum_{L=1}^{N} \frac{[f]^L}{2}$$

$$\left\{ 1 + \frac{(\xi_{ji}^L - 1)^2}{\sqrt{(\xi_{ji}^L - 1)^2 + 4\,\xi_{ji}^L \exp(\gamma_{ji}/RT)}} \right\} . \quad (5)$$

where

$$\xi_{ji}^L = \frac{[p_i]_{ex}}{[p_j]_{ex}} \cdot \bar{K}_{j \to i}^{\infty} \quad (6)$$

It is to be noted that this equation applies to all solutes, regardless of their electrical charge. In Equation 5, [p$_i$]$_{in}$ is the equilibrium concentration of the ith solute in moles/gram fresh cells. The first term on the right-hand side represents the solute in the cell water; the following terms represent the solute adsorbed on a total of N types of sites. [f]^L is the concentration of the Lth type of adsorption sites in moles per gram of fresh cells.

All permeant solutes are found in the cell water; the actual concentration of a particular solute, say the ith in the cell water varies with the concentration of the solute in the external medium ([p$_i$]$_{ex}$), the equilibrium distribution coefficient of the ith solute, q$_i$, between cell water and the external medium and the water content of the cell, α, in milliliters of water per gram of fresh cells. In a plot of the intracellular concentration of the ith solute in the cell water against the external concentration of the ith solute, the relation is rectilinear (fig. 1A); the slope of this straight line is equal to αq_i, which generally varies inversely with the size of the hydrated molecules or ion (Ling et al., '73).

The adsorption terms may or may not exist for any one particular solute. When they do, there may be a high degree of selectivity. This selectivity in absorption is reflected in the value of $\bar{K}_{j \to i}^{\infty}$, the intrinsic equilibrium constant between one particular solute, called the ith solute, and another, called the jth. The superscript ∞ describes the equilibrium constant, does not involve near-neighbor interaction terms and is intrinsic. The subscript $j \to i$ indicates direction of exchange reaction. Thus certain isolated β- and γ-carboxyl groups prefer to adsorb K$^+$ more than Na$^+$, because $K_{Na \to K}^{\infty}$ is a large number, i.e., between 10^2 and 10^3 (Ling and Bohr, '70). $K_{Mg \to K}^{\infty}$ might be even larger (see below). On the other hand, other sites may strongly adsorb Mg^{++} but have no affinity for either K$^+$ or Na$^+$. Methods for determining $K_{j \to i}^{\infty}$ and $-\frac{\gamma}{2}$ from experimental data were given by Ling and Bohr ('70).

When there is only one type of adsorption sites for the ith solute, Equation 5 simplifies into

$$[p_i]_{in} = \alpha q_i \, [p_i]_{ex} + \frac{[f]}{2}$$

$$\left\{ 1 + \frac{(\xi_{ji} - 1)^2}{\sqrt{(\xi_{ji} - 1)^2 + 4\,\xi_{ji} \exp(\gamma_{ji}/RT)}} \right\} . \quad (7)$$

where [f] is the concentration of the adsorption sites; ξ_{ji}, γ_{ji} describes the adsorption properties of this type of sites. Figure 1 illustrates how the total equilibrium distribution in liv-
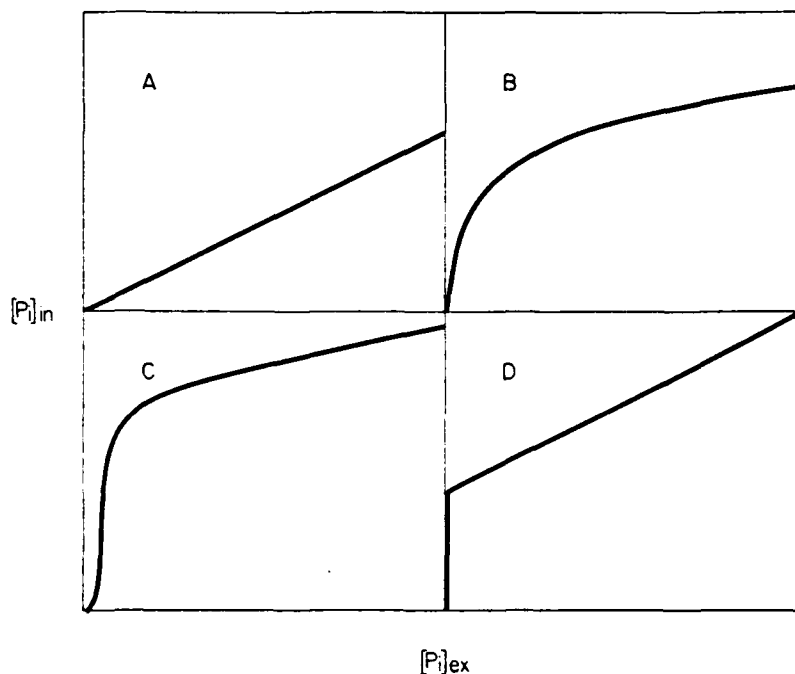
Fig. 1 Four types of theoretical curves of the concentration of solutes accumulated in living cells (ordinate = $[p_i]_{in}$) against the equilibrium concentration of the solute in the external bathing solution (abscissa = $[p_i]_{ex}$), derived according to Equation 4. A. Solute present only in cell water. B. Solute present both in cell water and adsorbed on one type of site showing no site-to-site interaction (i.e., Langmuir isotherm, $-\frac{\gamma}{2} > 0$). C. Solute present both in cell water and adsorbed on one type of adsorption site showing site-to-site cooperative interaction ($-\frac{\gamma}{2} > 0$). D. Solute present both in cell water and adsorbed on one type of site with very strong adsorption.

ing cells may vary with external solute concentration according to Equation 7. Figure 1A shows the case in which there is no adsorption, all solute being free in the cell water; figure 1B shows the case in which the solute is both free in the cell water and also adsorbed on one type of adsorption site that exhibits no site-to-site interaction (Langmuir adsorption isotherm, $-\frac{\gamma}{2} = 0$); figure 1C shows the case in which the solute is in the cell water and also adsorbed on sites that exhibit an autocooperative type of interaction among adsorption sites ($-\frac{\gamma}{2} > 0$); figure 1D shows the case in which the solute in the cell water is very strongly adsorbed on one type of site, which may be the Langmuir type or the autocooperative type. In figure 1D, in which the adsorption is clearly delineated, the slope of the straight-line portion of the curve, like that in figure 1A, is equal to the q-value (equilibrium distribution coefficient) of the solute in question,

tion, if the intracellular concentration is, as the extracellular concentration, expressed in moles per liter of cell water. The strong adsorption indicates a very large value of $\bar{K}^{\infty}_{j \to i}$, hence ξ^L_{ji}. Thus at very low values of $[p_i]_{ex}$ where $q_i [p_i]_{ex}$ is nearly zero, $\xi^L_{ji} >> 1$. As a result, the term in the large brackets equals 2 and $[p_i]_{in} = [f]^L$. Therefore the intercept on the ordinate is equal to the concentration of this type of strong binding sites.

According to the membrane theory, there is a mandatory interdependence among the distribution patterns of *all* permeant ions and the membrane potential Ψ. According to the association-induction hypothesis, there may be no interdependence at all between the bulk-phase distribution of one ion and another nor between bulk-phase ion distribution and Ψ. Interdependence in bulk-phase ion distribution, when it does exist, is limited to that between ions that share the same adsorption

sites and that between free cations and anions in the cell water that must exist either in an associated state or fully dissociated but must enter or leave the cell as neutral pairs or clusters. In this theory, the cellular resting potential depends on the nature and density of the *surface* sites and is thus not related directly to the concentration of the bulk-phase adsorption sites in the cytoplasm nor to the concentrations of their adsorbed ions, nor to the bulk-phase free ion concentrations, even though the chemical structures of the surface and bulk-phase fixed sites may be quite similar (Ling and Walton, '79).

It was suggested since the earliest version of the association-induction hypothesis presented in 1952 (see also Ling, '62; Ling and Ochsenfeld, '66) that the bulk of intracellular K$^+$ is adsorbed on the β- and γ-carboxyl groups of the intracellular proteins (Ling, '52; for recent repeatedly confirmed evidence, see Edelmann, '77, '78; Ling, '77b). Theoretical calculations demonstrated that these monovalent anionic sites can indeed preferentially adsorb K$^+$ over Na$^+$ (Ling, '62). A model system was used to demonstrate that isolated fixed carboxyl groups on nitrocellulose do indeed adsorb K$^+$ preferentially over Na$^+$; the same carboxyl groups exhibit no affinity for Mg^{++} (Ling, '67b). Carboxyl groups situated close together, on the other hand, behave quite differently. Thus carboxyl ion-exchange resins, in contrast to oxidized collodion, contain very high concentrations of fixed carboxyl groups (e.g., 1 to 2 M) and hence offer much greater chance for chelation and adsorption of divalent ions on pairs of carboxyl groups. It is not surprising that carboxyl ion-exchange resins preferentially adsorb divalent Ca^{++} and Mg^{++} over monovalent ions (Helfferich, '62).

Mg^{++}, like Na$^+$, is a highly hydrated ion. Thus, it can be deduced from the basic concept of the polarized multilayer theory of cell water and the theory of solute exclusion from water in the state of polarized multilayers that Mg^{++} should be excluded from cell water having a q-value substantially below unity. Although the association-induction hypothesis at this time offers no specific insight into the nature of the Mg^{++} adsorbing sites, it does state that these sites must be distinct from those isolated β- and γ-carboxyl groups on the muscle cell surface that prefer K$^+$ (Ling and Walton, '79); on this basis, *one can make the prediction that the accumulation of Mg^{++} in* the cell should offer no competition for K$^+$ adsorption, and vice versa. One should note that this prediction is diametrically opposed to that of the membrane theory.

MATERIALS AND METHODS

Most of these experiments were performed on isolated voluntary muscles and ovarian eggs of the North American leopard frogs (*Rana pipiens pipiens*, Schreber) which were force-fed canned dog food twice a week while kept in running water at room temperature. The four types of muscles used were sartorious, semitendinosus, tibialis anticus longus, and iliofibularis. Loose connective tissues were isolated from the legs and thighs of the same animals that provided the muscles. Ovarian eggs, 1.5-2 mm in diameter, were obtained from female frogs in late fall and winter. A cluster of about 20 to 30 eggs was used as a unit specimen for study.

Since this work was carried out over a period of several years, the procedures and compositions of solutions were continually improved on; those to be presented here were used in our latest and most definitive experiments.

The incubation solutions were modifications of the Ringer-GIB medium described in an earlier publication (Ling and Bohr, '69). Using this medium Ling and Bohr were able to maintain in physiological condition isolated frog muscles for at least a full week at 25°C as judged by the resting potential, K$^+$ and Na$^+$ contents as well as contractility.

In order to preserve the osmotic strength only approximately one half of the NaCl was replaced by MgCl$_2$ at two-thirds the molar concentration to obtain a maximum concentration of Mg^{++} without causing cell shrinkage; the other half was replaced by MgSO$_4$ at an equal molar concentration. In addition, the glucose concentration was reduced. This MgCl$_2$-MgSO$_4$ Ringer solution does not cause cell volume changes and was used in obtaining most of the data to be presented, although in earlier studies a simple MgCl$_2$ Ringer solution was used in which isoosmolar MgCl$_2$ replaced varying amounts of NaCl in the normal Ringer formula, and essentially similar results were obtained.

To prepare a Ringer's solution with different Mg^{++} concentrations in the bathing solution, two solutions were prepared and mixed in different proportions. The high-Mg^{++} solution

TABLE 1

Composition of high and low Mg++ Ringer solution used

	Stock solution conc. (M)	High Mg++ solution		Low Mg++ Solution	
		Vol. of stock solution (ml)	Final conc. (mM)	Vol. of stock solution (ml)	Final conc. (mM)
A MgCl₂	0.590	82.7	37.2	0	0
H₂SO₄	0.590	80	36.0	0	0
Sucrose	1.18	0	0	177	159
KCl	0.118	27.8	2.5	27.8	2.5
NaHCO₂.3	0.59	35.0	15.7	35.0	15.7
NaH₂PO₄	0.118	22.0	2.0	22.0	2.0
H₂O		936		922	
B CaCl₂	0.0845	15.6	1.0	15.6	1.0
Glucose	1.18	12.9	11.6	12.9	11.6
K-free GIB Medium	0.154	100		100	
Penicillin G, Na		(260 mg)	(0.2 mg/ml)	(260 mg)	(0.2 mg/ml)
Streptomycin		(260 mg)	(0.2 mg/ml)	(260 mg)	(0.2 mg/ml)
Total volume		1,312		1,312	

Part A and Part B of each solution was separately prepared, sterilized and equilibrated with 95% O_2 and 5% CO_2 to prevent Ca^{++} precipitation. Sterilization was by Millipore (0.2 μ pores) filtration.

contained 73.2 mM Mg^{++}, as a chloride and sulfate mixture (as mentioned above); the low-Mg^{++} stock solution contained only 0.06 mM Mg^{++}. It was decided that in order to obtain the most accurate results, alterations of the Mg^{++} concentration should not be accompanied by counteractive changes in the Na^+ concentrations. For this reason the low-Mg^{++} solution was prepared by replacing all the NaCl of a normal Ringer's solution with sucrose. However, trials showed that at equal osmolarity, substitution of sucrose for NaCl caused muscle shrinkage (table 1), whereas in a solution of sucrose with a concentration equal to 81% of the osmolarity of the NaCl used, cell volume remained constant. Thus, the latter concentration of sucrose was used to replace NaCl in the low-Mg^{++} stock solution.

Table 1 presents detailed information on the preparation of both the high and low Mg^{++} Ringer solutions. By mixing different volumes of stock solutions at the concentrations designated, two separate solutions A and B were prepared for each of the high and low Mg^{++} Ringer solutions. The A and B solutions were sterilized separately by passage through Millipore Filter (0.2 μm pore diameter) and then separately equilibrated with mixture of 5% CO_2 and 95% O_2 (also sterilized by passage through Millipore Filter) before the A and B components were finally mixed. It should be noted that one component of Solution B, the K-free GIB medium, was a chemically defined culture medium obtainable from Grand Island Biological Company, Chagrin Falls, Ohio. This medium contains besides 21 amino acids, 14 vitamins, etc., also NaCl (116 mM), Na_2HPO_4 (14.8 mM), $Ca(NO_3)_2$ (0.13 mM), $MgSO_4$ (0.8 mM), glucose (6.67 mM) and 10 mg/liter of phenol red (which should remain a salmon pink, corresponding to a pH of about 7.4). It is from the GIB medium that both the high and low Mg^{++} Ringer solutions received an equal amount of NaCl to yield a final concentration of 8.86 mM. Thus in all solutions used this same concentration of NaCl was present. We determined Mg^{++}, Na^+, and K^+ concentration with a Perkin-Elmer Model 103 atomic adsorption spectrophotometer; the procedure was that earlier described (Ling and Bohr, '69). To extract these ions, the muscle tissues were heated for 20 minutes in 3 ml of 0.1 HNO_3 in a covered Nalgene tube in a boiling bath. After centrifugation, the clear supernatant solutions were diluted with a universal "radiation buffer." The diluted sample solution as well as the standards contained 97 mM LiCl, 30 mM $NH_4H_2PO_4$, and the same concentration of HNO_3.

Tissue chloride concentrations were determined in the 0.1 N HNO_3 muscle tissue extracts by coulometric titration with a Buchler-Cotlove chloridometer.

To eliminate one extraneous source of Mg^{++}, the extracellular space fluid was removed from the frog muscle, using the centrifugation
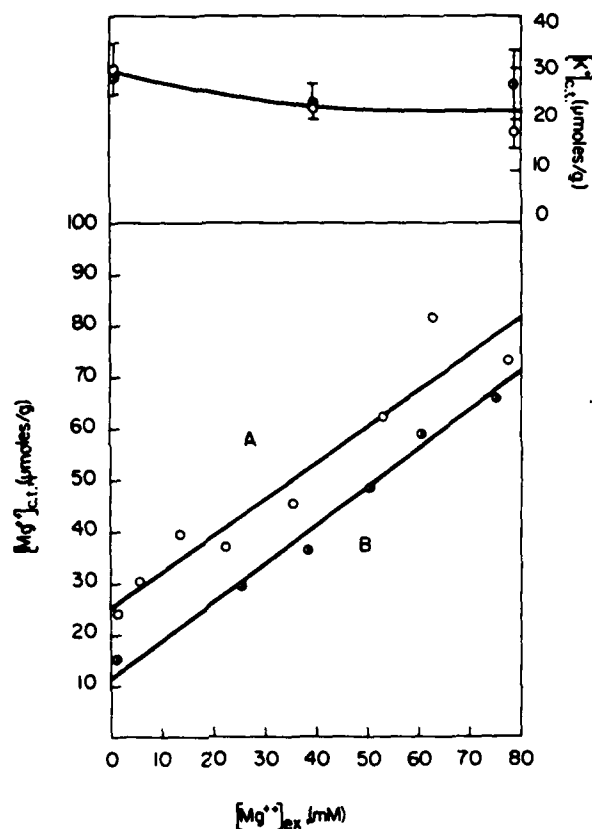
Fig. 2 Equilibrium Mg^{++} and K$^+$ distribution in isolated connective tissues at 25°C. Incubations were 96 hours (5°C) (A) and 17.5 hours (25°C) (B) in 100 ml of solute with shaking. The connective tissues (c.t.) were centrifuged to remove extraneous fluids before extraction of and analysis for Mg^{++} and K$^+$.

procedures described by Ling and Walton (Ling and Walton, '75a). Another extraneous source is Mg^{++} adsorbed or otherwise entrapped in the connective tissue elements (i.e., loose connective tissues, small nerves, blood vessels, etc.) present in the isolated muscles. To make proper corrections we also studied the Mg^{++} uptake of "pure" connective tissue elements isolated from the same frogs and exposed to the same bathing solutions. Figure 2 shows that the Mg^{++} and K$^+$ distribution patterns of these connective tissue elements closely resemble those in frog muscle tissue (see figs. 5 and 6 below) except that the rectilinear part of the Mg^{++} curve is much steeper, with slopes of 0.71 (5°C) and 0.84 (25°C). These data and an estimated 4.3% for the weight of connective tissues in centrifuged

muscles (Ling and Walton, '75b) provided the basis for correction of the connective tissue contributions.

To calculate the intracellular Mg^{++} concentration, [Mg^{++}]$_{in}$, from Mg^{++} concentration in centrifuged muscle and connective tissues, [Mg^{++}]$_{cell + c.t.}$ in units of μmoles per gram of fresh weight, the following equation was used:

$$[Mg^{++}]_{in} = \frac{[Mg^{++}]_{cell + c.t.} - 0.043\,[Mg^{++}]_{c.t.}}{(1 - 0.043)} =$$

$$\frac{1.04\,[Mg^{++}]_{cell + c.t.} - 0.043\,[Mg^{++}]_{c.t.}}{} \quad (6)$$

[Mg^{++}]$_{in}$ is the Mg^{++} concentration in the cell in μmoles/g of fresh cells. [Mg^{++}]$_{c.t.}$ is the concentration of Mg^{++} in one gram of fresh connective tissues; 0.043 is the percentage weight of the wet connective tissues. Similar formulas were used for calculating [K$^+$]$_{in}$ and [Cl$^-$]$_{in}$.
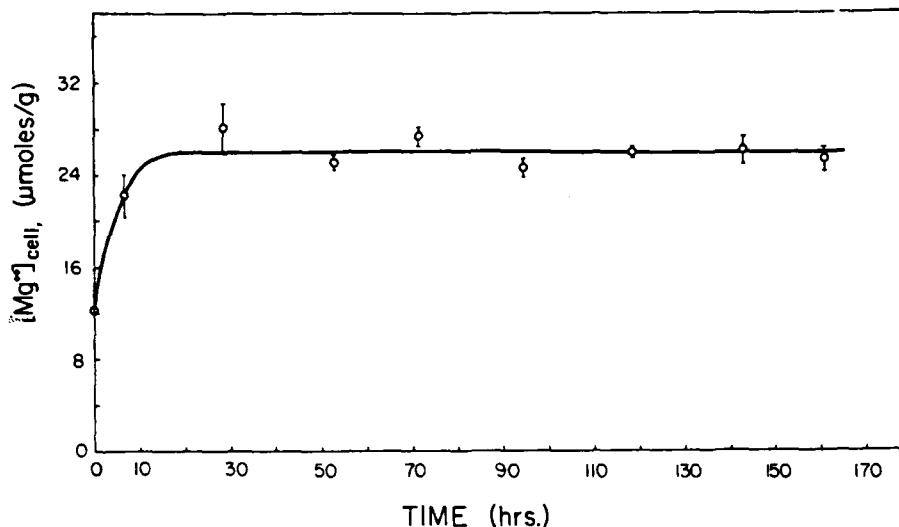
Fig. 3   Time course of Mg⁺⁺ uptake by frog voluntary muscles at 5°C. Muscles of all four types described under MATERIALS AND METHODS were incubated at 4°C in 100 ml of high-Mg⁺⁺ Ringer's solution (Mg⁺⁺ concentration = 73.2 mM). Four muscles, one of each kind, were placed in separate flasks and taken out at intervals. Before extraction and analyses the muscles were centrifuged to remove extracellular fluids.

RESULTS

Time course of Mg⁺⁺ uptake by frog muscles at 4°C

Frog muscles were incubated at 4°C in a modified Ringer-GIB medium containing 73.2 mM Mg⁺⁺. At intervals, four muscles, one each of the four types mentioned earlier, were removed, centrifuged to remove the extracellular fluids and then analyzed for Mg⁺⁺. Figure 3 shows that at this temperature, the intracellular Mg⁺⁺ concentration rose from a value of 12.3 ± 0.40 μmoles per gram of fresh muscle cells to 26.0 μmoles/gram within 29 hours and was maintained with little change at this level for at least one week. It must be mentioned that this set of experiments was conducted primarily to find out an adequate incubation time period to assure equilibrium distribution of Mg⁺⁺ in the tissues and not to accurately assess the exact time for Mg⁺⁺ to reach equilibrium. It is entirely likely that it took considerably less than 29 hours for the equilibrium to be reached. In experiments conducted between 23° to 26°C, Gilbert reported that "within three hours, equilibrium was reached ..." (Gilbert, '60: p. 1105). His figure (fig. 1: p. 1106) suggested that equilibrium could have been reached even earlier, i.e.,

in an hour and henceforth maintained unchanged for at least 72 hours. Figure 4 shows our own time course of Mg⁺⁺ uptake equivalent to figure 3 but at 25°C and within considerably more experimental points at earlier times. The data clearly showed that in only 20 minutes, Mg⁺⁺ has already reached an equilibrium level and was then maintained for as long as the experiment lasted (6 hours). Considering that in our experiments as well as those of Gilbert the same mixed muscles were used including thick ones (ileofibularis, semitendinosus, and tibialis anticus longus) with diameters usually about 2 mm wide, the permeability of frog muscle cells to Mg⁺⁺ must be very high indeed. Other data not shown confirmed Gilbert's finding that the equilibrium Mg⁺⁺ concentration was maintained at a constant level for at least 48 hours. These time courses provided the basis for studying the steady levels of Mg⁺⁺ in the muscle cells to be described next.

The steady levels of Mg⁺⁺ in muscle cells at varying external Mg⁺⁺ concentrations

That the steady levels of Mg⁺⁺ concentration in frog muscles increase rectilinearly with increasing external Mg⁺⁺ concentration
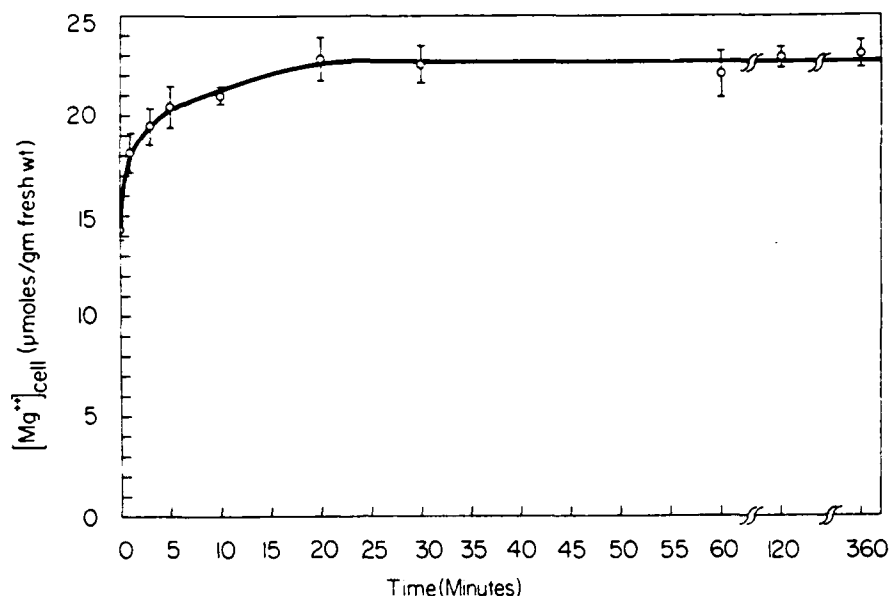
Fig. 4 Time course of Mg⁺⁺ uptake by frog voluntary muscles at 25°C. Except for the difference in temperature, the details of the procedure used is the same as described under figure 3.

was discernible in the data of Conway and Cruess-Callaghan ('37) and was clearly shown by Gilbert ('60) up to an external Mg⁺⁺ concentration of 18 mM. Only in our studies was the range of external Mg⁺⁺ concentration considerably extended, and the centrifugation method was used to eliminate Mg⁺⁺ in the extracellular fluid and Mg⁺⁺ and K⁺ uptake in loose connective tissues were used to make correction for the contributions from "connective tissue elements" in the muscle tissues.

Mg⁺⁺ concentrations in muscle cells at 5°C and 25°C are presented in figures 5 and 6, respectively, in micromoles per gram of fresh cells and are plotted against the external Mg⁺⁺ concentrations. The slopes of the rectilinear parts of these curves obtained by the method of least squares are 0.220 and 0.206 for the 5°C and 25°C experiments, respectively. The relation of intra- and extracellular equilibrium Mg⁺⁺ concentration in these muscle cells is basically similar to that seen in the connective tissue shown in figure 2, except the slopes of the rectilinear curve in figure 2 are much higher.

Also to be noted are the higher Mg⁺⁺ contents (12 to 15 mmoles/kg) in the presence of normal external Mg⁺ concentration observed

here than those reported by Gilbert ('60) (6.80 mmoles/kg), and by Sheid and Straub ('68) (8.8 mmoles/kg). However, our values are close to the figure given by Conway and Cruess-Callaghan (11.0 mmoles/kg). One possible reason for the differences could be the fact that to the best of our knowledge, none of the other authors fed their frogs, while ours were. This suggestion agrees with Gilbert's observation that winter frog has less Mg⁺⁺ than summer frogs since, generally speaking, winter frogs tended to be kept for longer periods of time without foods.

Independence of K⁺ concentration in muscle exposed to varying concentrations of external Mg⁺⁺

Figures 5 and 6 also show the concentrations of K⁺ in the same muscle cells that steadily gained in Mg⁺⁺ concentration in response to the increase of external Mg⁺⁺ concentration. It is clear that with the increase of external Mg⁺⁺ concentration from 1.2 mM to 73.2 mM, the intracellular K⁺ concentration remained within the limits of experimental error, entirely constant at about 90 μmoles/gram of fresh cells. These findings were quite reproducible as long as the muscles were
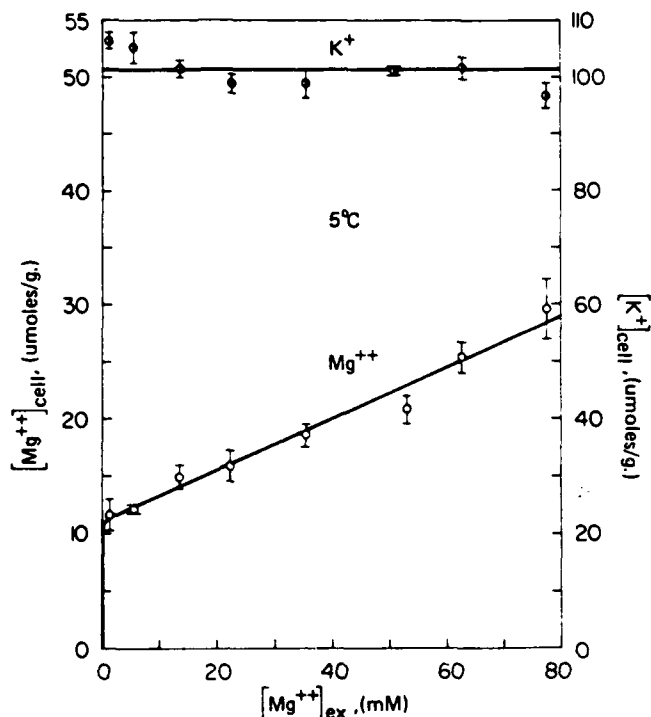
Fig. 5 Equilibrium Mg^{++} and K$^+$ distribution in frog voluntary muscles at 5°C. Incubation was in a 5°C shaking water bath for 96 hours. The muscles were centrifuged to remove extracellular fluids before extraction of and analysis for Mg^{++}. Each point is the average of four determinations; the distance between the horizontal bars is twice the standard error. The solid line intersecting most of the Mg^{++} data points is theoretical according to Equation 1. The numerical values used are $\alpha = 0.78$, $q_{Mg} = 0.264$, $K^{00}_{Mg} = 10^{-4}M$, $[f] = 11.0$ μmoles/g fresh muscle cells, and $-\frac{\gamma}{2} = 0$ Kcal/mole.

maintained in good shape. Damage of the cells by bacterial contamination for example, caused loss of K$^+$ and gain of Mg^{++} when the muscles were incubated in a high Mg^{++} Ringer.

Influence of increasing external Mg^{++} concentration on distribution of K$^+$ and Mg^{++} in frog ovarian eggs

Figure 7 shows that, as in the case of frog muscles, increasing external Mg^{++} from 1.2 mM to 73.2 mM brought about a rectilinear increase of intracellular Mg^{++} in frog ovarian eggs. The intercept of the straight portion of the curve on the ordinate is 19.4 moles/g, while the slope of the same part of the curve is roughly 0.31.

Figure 7 also shows that, as in the case of

frog muscle, increasing external Mg^{++} concentration from 1.2 to 73.2 produced no drastic change of the intracellular K$^+$ concentration in frog eggs. In spite of considerable scattering, the data generally agree with those shown in figures 5 and 6 for frog muscles.

Chloride concentration in muscles in the presence of increasing concentrations of external MgCl$_2$ and MgSO$_4$ at 25°C

The Cl$^-$ concentrations in muscles which provided the data of figure 4 are shown in figure 8 against external Mg^{++} concentration and in figure 9 against total external Cl$^-$ concentration. Like Mg^{++}, Cl$^-$ also showed a steady gain of intracellular concentration with an increase in the external concentration of MgCl$_2$. The experimental points of the Cl$^-$
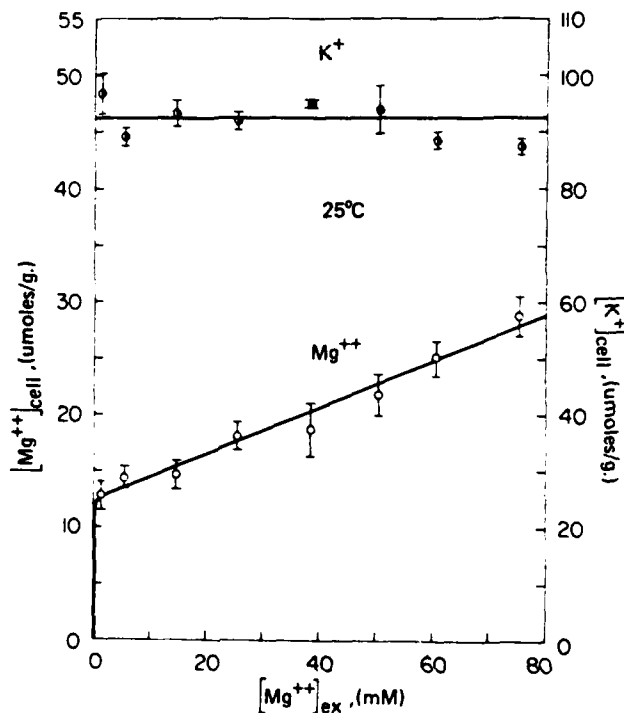
Fig. 6 Equilibrium Mg⁺⁺ and K⁺ distribution in frog voluntary muscles at 25°C. Incubation was in a 25°C shaking water bath for 17.5 hours. Muscles were centrifuged to remove extracellular fluids before extraction of and analyses for Mg⁺⁺. Each point is the average of four determinations; the distance between the horizontal bars is twice the standard error. The solid line intersecting most of the Mg⁺⁺ data points is theoretical according to Equation 1. The numerical values used are $\alpha = 0.78$, $q_{Mg} = 0.280$, $K_{Mg}^{00} = 10^{-4}M$, [f] = 12.3 μmoles/g fresh muscle cells, and $-\frac{\gamma}{2} = 0$ Kcal/mole.

curve of figures 8 and 9 like the Mg⁺⁺ data in figures 5 and 6, have also been corrected for the contribution of Cl⁻ from the connective tissue elements. The corrected data of muscle cell Cl⁻ yielded, by the method of least squares, a slope of 0.220, which has essentially the same value as the slope of the Mg⁺⁺ curve in the same muscles (i.e., 0.206).

Effect of varying external K⁺ concentration on intracellular Mg⁺⁺ concentration

Figures 5, 6, and 7 show that accumulation of Mg⁺⁺ in muscle and egg cells had no effect on the concentration of intracellular K⁺; table 2 shows that the converse was also true in frog muscle. By the addition of solid KCl to either a normal Ringer-GIB medium or to a modified Ringer-GIB solution with the NaCl replaced by an equiosmolar quantity of sucrose, the external K⁺ concentration was increased from

2.77 to approximately 100 mM; as a result of 18.5 hours of incubation at 25°C, the intracellular K⁺ concentration doubled. Yet in spite of this large gain of intracellular K⁺ concentration, the intracellular/extracellular ratio of Mg⁺⁺, after having been corrected for the difference in weight changes, remained unaffected, as shown in the last column of table 2.

DISCUSSION

The membrane theory

As discussed in the THEORIES section, the membrane theory predicts that extensive changes in external Mg⁺⁺ concentration will have profound effects on K⁺ and Cl⁻ distribution in living cells and that extensive changes in external K⁺ will have profound effects on Mg⁺⁺ distribution. However, our experimental observations do not substantiate these predictions. Quite the contrary, in the concentration
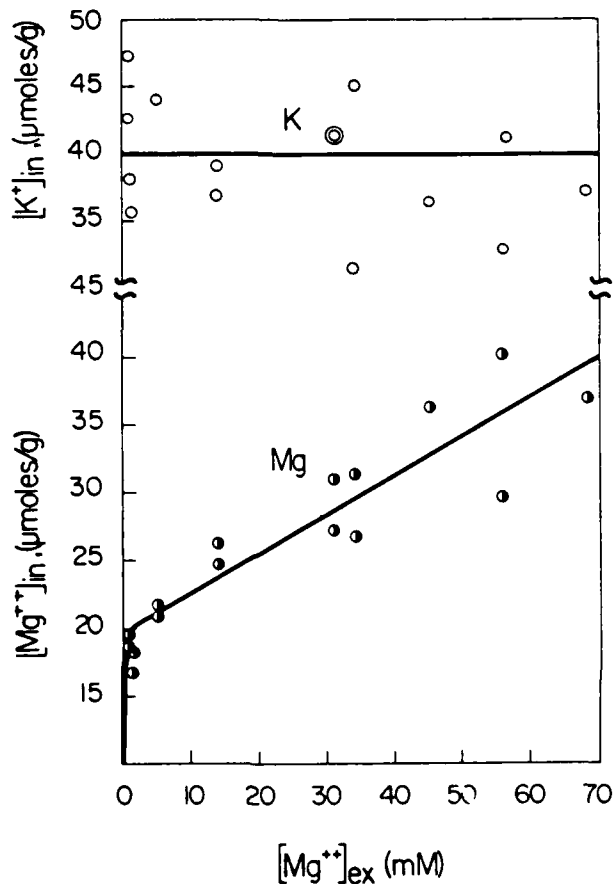
Fig. 7 Equilibrium Mg⁺⁺ and K⁺ distribution in frog ovarian eggs. The experiment was carried out in winter. Twenty to thirty mature ovarian eggs were treated as a group. Incubation was at 4°C with very gentle shaking for two days. Eggs were blotted before extraction of and assay for Mg⁺⁺ and K⁺. Each point represents a single experiment . The solid curve intersecting most of the Mg⁺⁺ data points is theoretical according to Equation 1. The numerical values used are $\alpha = 0.313$, $q_{Mg} = 0.61$, $K_{Mg}^{\infty} = 5 \times 10^{-3}M$, $[f] = 19$ μmoles/g fresh egg cells, and $-\frac{\gamma}{2} = 0.5$ Kcal/Mole.

range investigated the accumulations of K⁺ and of Mg⁺⁺ appear to be independent of each other. We conclude that living muscle cells do not follow Donnan's theory of membrane equilibrium.

It should be mentioned that although it was not explicitly stated by Gilbert that the Donnan theory of membrane equilibrium was not followed in the Mg⁺⁺ distribution in frog muscle cells, by postulating a Mg⁺⁺ pump such a conclusion was clearly implied. Gilbert's implication, of course, agreed with an earlier conclusion of Ling that the distribution of Mg⁺⁺ (as well as other solutes) in frog muscles

do not follow the prediction of Donnan's theory of membrane equilibrium (Ling, '55, '62).

Gilbert ('60) calculated from his data that such a Mg⁺⁺ pump postulated would consume a minimal amount of energy, i.e., 1% of the total resting cell energy production rate. We disagree with his conclusions but will present the entire argument in a later paper. One specific point however can be discussed to illustrate one facet of this disagreement.

Gilbert's calculation was based on the assumption that of the three fractions of effluxing Mg⁺⁺, the slowest (lasting 300
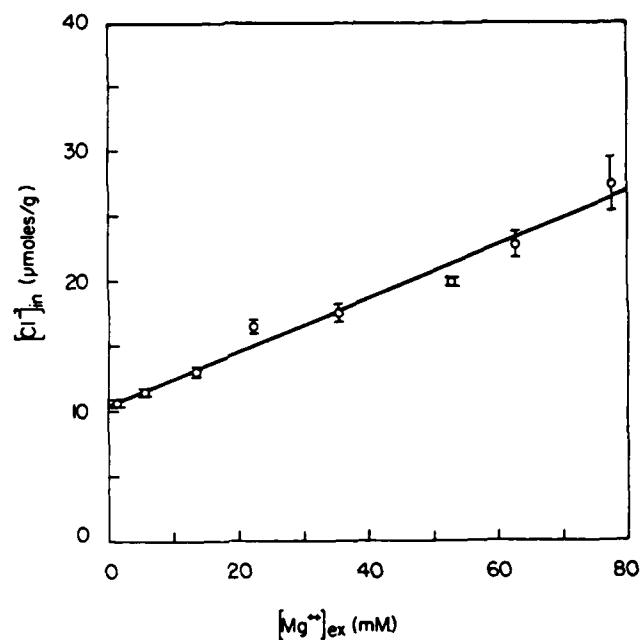
Fig. 8   Equilibrium distribution of Cl^- in frog voluntary muscles at 5°C plotted against equilibrium external Mg^{++} concentration. Data were from the same muscles shown in figure 4, were corrected for chloride in connective tissues, and are expressed in μmoles per gram of fresh muscle cells. Each point is the mean of four determinations ± S.E.
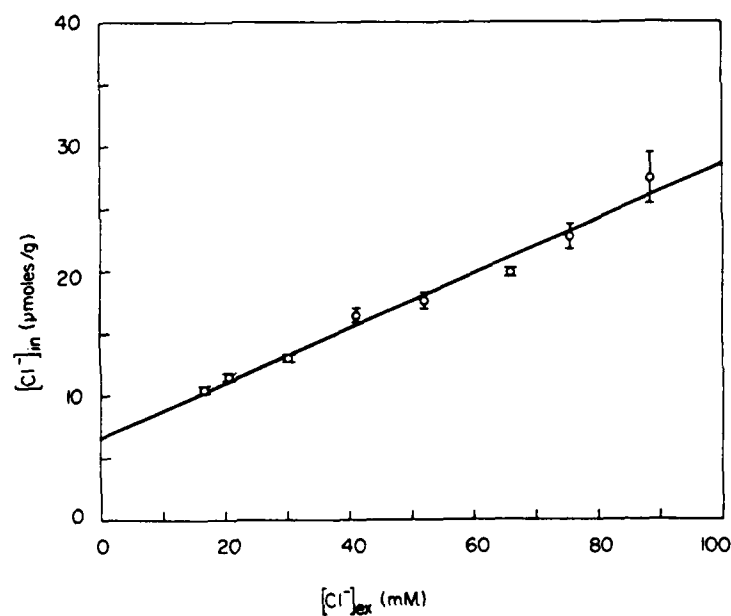

Fig. 9   Equilibrium distribution of Cl^- in frog voluntary muscles at 5°C plotted against external Cl^- concentration. Data are the same as in figure 7 but are plotted differently. Each point represents means of four determinations ± S.E. By the method of least squares, the slope of the curve is 0.220.

TABLE 2

Effect of varying K^+ concentration upon intracellular concentration

		W_f/W_i	$[K^+]_{ex}$	$[K^+]_{in}$	$[Mg^{++}]_{ex}$	$[Mg^{++}]_{in}$	$\dfrac{[Mg^{++}]_{in}}{[Mg^{++}]_{ex}}$	$\dfrac{[Mg^{++}]_{in}}{[Mg^{++}]_{ex}}\dfrac{W_f}{W_i}$
			(mM)	*(μmoles/q)*	*(mM)*	*(μmoles/q)*		
I	Control	0.97±0.03	2.91	90.9±3.4	0.88	9.24±0.53	10.50±0.61	10.20±0.62
	Experiment	1.00±0.03	94.10	186.0±2.7	0.82	8.39±0.60	10.20±0.07	10.20±0.71
II	Control	0.99±0.02	2.71	93.5±2.9	1.08	9.16±0.75	8.50±0.70	9.02±1.12
	Experiment	0.98±0.03	96.70	193.3±5.0	0.84	8.64±0.68	10.30±0.81	10.00±0.73
III	Control	0.99±0.02	2.76	93.1±3.3	1.08	9.81±0.11	9.33±0.16	9.24±0.34
	Experiment	0.78±0.02	113	193.7±8.4	0.98	12.00±0.72	12.20±0.72	9.42±0.33
IV	Control	1.00±0.01	2.71	77.8±6.2	1.00	8.68±0.76	8.67±0.75	8.68±0.66
	Experiment	0.79±0.02	106	168.8±7.8	0.89	9.64±0.66	10.90±0.76	8.96±0.29

The first column gives the ratio of final weights of the muscles over the initial weights. The last column represents the intracellular/extracellular ratio of Mg^{++} after corrections for weight change. In Experiments I and II, KCl was added to a normal Ringer's solution; in Experiments III and IV, KCl was added to a Ringer's solution in which NaCl was replaced by isoosmolar concentrations of sucrose, which led to marked shrinkage (see text under MATERIALS AND METHODS). In each case, data were averages from four determinations ± S.E.

minutes) corresponded to that of pumping. We disagree with Gilbert on this issue and believe that it is a faster fraction corresponding probably to what Gilbert called the "second phase" lasting "about 30 minutes" and which Gilbert attributed to extracellular water and connective tissue, as the one which actually represents efflux in intracellular free Mg^{++}. For this reason, we believe the hypothetical Mg^{++} pump would consume much more than a trivial amount of energy.

The living cell is a functionally coherent unit; in an energy-balance consideration, like any budget balancing consideration, it is of less significance unless all energy expenditures are considered at once and the total compared with the available energy. With this in mind, one recalls that it has been repeatedly shown that one of the pumps postulated, the Na^+ pump, would consume much more energy than the frog muscle cell commands (Ling, '62; Ling et al., '73; Jones, '65; Minkoff and Damadian, '73). Several remedial theories have been put forth to explain this contradiction.

A. Exchange diffusion

It was suggested that if "exchange diffusion" (a one-to-one, Na^+-for-Na^+ exchange between the intracellular phase and extracellular phase) accounts for a substantial portion of the total Na^+ efflux rates, the energy requirement of the pump might be significantly reduced to a level compatible with the amount of energy available (Ussing, '49). However, experiments conducted to confirm the existence of such exchange diffusion have repeatedly produced negative findings (Ling, '55; Hodgkin and Keynes, '55; Buck and Goodford, '66; Hoffman and Kregenow, '66; Ling and Ferguson, '70).

B. Na^+ sequestration in the sarcoplasmic reticulum

Another postulation was the idea that the bulk of Na^+ in muscle cells is not truly *inside* the cell but is sequestered *outside* the cell, in the sarcoplasmic reticulum (SR) (Keynes and Steinhardt, '68; Zierler, '72; Rogus and Zierler, '75; Freedman, '73). As in the case of the exchange diffusion theory, considerable anatomical and physiological evidence refuting such a postulation had already existed before the postulation was made (Ling, '73), and additional contradicting evidence was found after it was made (Ling and Walton, '75b). Recently, with the aid of electron probe microanalysis, Somlyo et al. showed that Na^+ is evenly distributed in muscle cells and is not sequestered in the SR (Somlyo et al., '77).

C. Only net transfer of Na^+ needs energy

Still more recently, Glynn suggested that in calculating the energy requirements of the Na^+ pump, only the net transfer of the Na^+ need be considered (Glynn, '77). The new idea that Glynn now suggests is that the inward leakage of Na^+ is channeled into a generator to produce energy for the outward pumping. Indeed, according to this mechanism, this generator is nothing more than the Na pump

itself, i.e., the Na$^+$-K$^+$ activated ATPase functioning in reverse (Lew et al., '70). Therefore, as long as the level of Na$^+$ in the cell does not change, the energy requirement for outward pumping is altogether eliminated; for each mole of Na pumped out, the energy requirement is quantitatively replaced by the same amount of energy regenerated by one mole of Na$^+$ going into the cells.

This concept seems unlikely, considering that it is in fact yet another version of the "Maxwellian demon." A more definitive conclusion can also be deduced from an experiment designed to test it. The experiment was based on another main principle of the Na pump theory, i.e., the cardiac glycoside ouabain, at the proper concentration, specifically poisons and thus inactivates the Na pump. Therefore, if energy is regenerated through the harnessing of all inwardly diffusing Na$^+$, inward movement of Na$^+$ should also be greatly slowed down when the cell is poisoned with ouabain. In fact Horowicz and Gerber ('65) have shown that inward movement of Na$^+$ was not altered by exposure to ouabain. Therefore the inward movement of Na$^+$ cannot be mediated by the Na pump nor can it provide the energy to support its outward pumping.

Since none of the "remedies" proposed has discounted the fact that there is not enough energy to run the Na pump alone, introducing still another energy-consuming pump for Mg^{++} cannot be considered a reasonable solution to this basic contradiction of the membrane theory. In addition, the membrane theory cannot explain (1) why an increase of external Mg^{++} concentration has no effect on the resting potential, as we have recently observed (Ling and Walton, '79); (2) why the behavior of chloride in the cell is so different from that predicted by the membrane equilibrium theory; nor (3) why a large increase in intracellular Mg^{++} concentration causes no change in intracellular K$^+$ concentration.

The observations reported here offer compelling reasons to seek answers along other lines of thought.

The association-induction hypothesis

The lack of correlation between the experimental findings and the results predicted according to the membrane theory contrasts sharply with the correlation between these same findings and results predicted according to the association-induction hypothesis.

A. The existence of two types of ions in living cells: free and adsorbed

The association-induction hypothesis like Troschin's sorption theory ('65) predicts that there are potentially two major kinds of any intracellular ions—one (or more) saturable adsorbed fraction and an unsaturable fraction distributed in the cell water. The saturable adsorbed fraction may exhibit characteristics demonstrable by any one of a variety of adsorption isotherms as shown in figures 1A to 1D. Each of these cases has now been demonstrated: 1A as in methanol, D-ribose distribution in frog voluntary muscles (Ling and Will, '69); 1B as in D-glucose distribution in insulinized frog muscle at 0°C (Ling et al., '69); 1C as in K$^+$ and Na$^+$ distribution in frog muscle (Ling, '66; Ling and Bohr, '71; Ling and Ochsenfeld, '73), and in mammalian tissues (Jones and Karreman, '69; Gulati and Jones, '71; Jones, '70); 1D as in Mg$^+$ distribution in frog muscle and egg described in this paper.

The rectilinearity of the Mg^{++} distribution curves in figures 2-6 suggests that full saturation of the adsorbed sites appears to have been reached at an external Mg^{++} concentration of only 1 mM (more direct evidence will be presented in a companion paper, Ling and Walton, '79). This indicates that the equilibrium constant K_{Mg} is at least as high as 10^4 M^{-1} and that this value was used in the construction of the theoretical curve shown in figures 4 and 5.

B. The independence of Ψ and external Mg^{++} concentration

As shown in preceding publications (Ling, '59, '60, '62, '67a,b, '75, '78b) the resting potential is consistent with the theory that it is determined by the nature and density of fixed anions on a macroscopically thin surface layer of the cell. The gross intracellular cation distribution, on the other hand, depends on the q-value of the cation in the cell water and on the average concentration of fixed anionic sites in the bulk cytoplasmic phase. No causal relation between Mg^{++} concentration in the cell and the resting potential was expected; none was found (Ling and Walton, '79).

C. The independence of K$^+$ accumulation and Mg^{++} accumulation

The data presented here clearly show that the adsorption sites for K$^+$ and Mg^{++} are quite different. There is already evidence that the

K$^+$-preferring bulk-phase intracellular sites as well as surface anionic sites are the β- and γ-carboxyl groups of proteins (Ling and Ochsenfeld, '65; Ling, '77b; Edelmann, '77, '78).

D. The rectilinear relation between $[Mg^{++}]_{in}$ and $[Mg^{++}]_{ex}$ and between $[Cl^-]_{in}$ and $[Cl^-]_{ex}$.

Since virtually all the specific Mg^{++} adsorption sites are fully or almost fully occupied at an external Mg^{++} concentration of 1 mM, the linear gain of intracellular Mg^{++} was most probably due to Mg^{++} in the cell water. As such this gain is described by the first term on the right-hand side of Equation 5. The Mg$^+$ entering the cell was accompanied by an equivalent concentration of the principal anions, chloride and sulfate. The fact that the rectilinear portion of the Mg^{++} curve and the rectilinear Cl$^-$ curves have similar slopes (0.21 and 0.22) further affirms this view.

E. The q-value for Mg^{++} (salts) and the mechanism for its exclusion from cell water

Owing to the tightness of Mg^{++} adsorption and the apparent saturation of Mg^{++} adsorption sites at very low external Mg^{++} concentration, the data presented in this paper offer unusual opportunities to estimate the q-value for Mg^{++} in the cell water of frog voluntary muscle. On the assumption that the linear portion of this curve in a plot of intracellular vs. extracellular Mg^{++} concentration represents gain only in Mg^{++} in the cell water, the q-value is simply the slope divided by the water content of the cell. Thus for a frog ovarian egg with a water content of 51.3% (Ling and Ochsenfeld, '77) the q-value for Mg^{++} is $0.31/0.51 = 0.61$. For frog voluntary muscle, the water content of centrifuged muscle is 78%, and the q-value for Mg^{++} is $0.220/0.78 = 0.281$ at 5°C and $0.206/0.78 = 0.264$ at 25°C.

According to the van't Hoff equation,

$$\Delta H = RT^2 \frac{d^{ln} q}{dT}. \tag{8}$$

where ΔH, the enthalpy of distribution and q is as earlier defined. For temperatures not too far apart, ΔH may be assumed to be constant. Under this assumption Equation 8 can be integrated:

$$\Delta H = \frac{R}{\frac{1}{T_2} - \frac{1}{T_1}} \, ln \, \frac{q_2}{q_1}. \tag{9}$$

Inserting our experimental data, we have

$$\Delta H = \frac{1.987}{\frac{1}{278} - \frac{1}{298}} \, ln \, \frac{0.281}{0.264}, \tag{10}$$

$$= -516 \text{ cal/mole.}$$

From the relationship

$$\Delta F = - R\bar{T}^{ln} \, \bar{q}, \tag{11}$$

where $\bar{T}$ is the average of T_1 and T_2 and $\bar{q}$, the average of q_1 and q_2. Therefore

$$\Delta F = - 1.987 \, \frac{278 + 298}{2} \, ln \, \left(\frac{0.281 + 0.264}{2} \right) \tag{12}$$

$$= 744 \text{ cal/mole.}$$

Now

$$\Delta F = \Delta H - \bar{T}\Delta S, \tag{13}$$

from which we obtain

$$\Delta S = \frac{\Delta F - \Delta H}{\bar{T}} = \frac{744 - (-516)}{288} \tag{14}$$

$$= 4.37 \text{ cal/deg/mole.}$$

These data show that the enthalpy is moderately favorable for Mg^{++} accumulation in the cell water and that it is primarily an unfavorable entropic mechanism that accounts for the partial exclusion of Mg^{++} salt from the cell water (Ling, '65b; Ling and Sobel, '75).

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